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RP-HPLC separation of the diastereomers of technetium-99m labelled tropanes and identity confirmation using radio-LC-MS

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Abstract

^{99m}Tc-TRODAT-1 (technetium(V)-oxo-2-[[2-[[[3-(4-chlorophenyl)-8-methyl-8-azabicyclo[3.2.1]oct-2-yl]methyl](2-mercaptoethyl)amino]ethyl]]amino]-ethanethiolato(3-)) and ^{99m}Tc-TRODAT-M, the 4-methylphenyl derivative of ^{99m}Tc-TRODAT-1, are at this moment being evaluated in clinical trials as imaging agents for the central nervous dopamine transporter system. Both compounds are formed as a mixture of two major diastereomers. As the tracer concentration in preparations for clinical investigations is very low (30–150 pmol/ml), identification of these ^{99m}Tc-complexes was, up to now, carried out indirectly using X-ray diffraction analysis of the corresponding rhenium complexes which can be synthesized in gram amounts. In this study, we developed a convenient and practical reversed phase HPLC-method for purification and isolation of the respective diastereomers of ^{99m}Tc-TRODAT-1 and three of its derivatives using mixtures of solvents which are compatible with biological studies, i.e. aqueous buffers and ethanol. Furthermore, direct identity confirmation of the ^{99m}Tc-complexes using radio-LC-MS was successfully elaborated.

Keywords: Technetium-99m-oxo; Tropanes; Diastereomers; Reversed phase HPLC; Radio-LC-MS

1. Introduction

Patients with Parkinsonian syndromes are characterised by a severe loss of dopamine neurons and dopamine transporters expressed on them. Tropane derivatives, such as cocaine, show affinity for the central nervous dopamine transporter system. Radiolabelled agents that bind to the dopamine transporter can be used to visualise dopaminergic dysfunction in Parkinsonian syndromes and differentiate between Parkinsonian syndrome and other degenerative diseases with comparable clinical symptoms, such as essential tremor. Several

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	X	Y
^{99m}Tc-TRODAT-1	Cl	H
^{99m}Tc-TRODAT-M	CH_3	H
^{99m}Tc-TRODAT-DM	CH_3	CH_3
^{99m}Tc- TRODAT-E	CH_2-CH_3	H

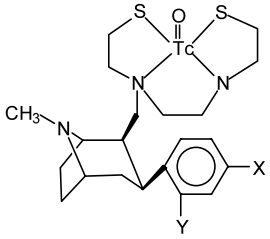

Fig. 1. Structure of ^{99m}Tc-TRODAT-1 and three of its derivatives.

iodine-123 labelled tropane derivatives have been evaluated as potential tracer agent for such investigations [1] and one of these (ioflupane (DaTSCAN®, Amersham Health, UK)) is now commercially available [2]. The high cost and the non-continuous availability of iodine-123 have encouraged the search for a ^{99m}Tc-labelled alternative. Technetium-99m is the preferred radioisotope for scintigraphic imaging because of its continuous availability at reasonable cost from a ^{99}Mo/^{99m}Tc-generator and its ideal radiation characteristics. Kung et al. have proposed ^{99m}Tc-TRODAT-1 [3] and ^{99m}Tc-TRODAT-M [4] (Fig. 1) as alternatives for iodine-123 labelled tropane derivatives and these agents are now being evaluated in clinical trials. In both ^{99m}Tc-labelled agents, a technetium binding bisamino bisthiol (BAT) tetraligand is coupled to a tropane moiety. Upon labelling with ^{99m}Tc, a Tc(V)oxo core is bound by the two nitrogen atoms and two thiol sulfur atoms to form a neutral complex. Because of the presence of chiral carbon atoms in the tropane moiety and an asymmetric nitrogen atom in the tetraligand, several diastereomers can be formed of which two are prominent [5]. Kung et al. were unable to separate these diastereomers on a polymer based reversed phase column. A reasonable separation was achieved on a chiralpak AD column, eluted with hexane–ethanol (3:1, V/V) at a flow rate of 1 ml/min after isolation of the diastereomeric mixture on a PRP-1 column eluted with acetonitrile/dimethylglutaric acid buffer pH 7 (80:20, v/v) at a flow rate of 1 ml/min [5]. This mixture of organic solvents is far from optimal for biological studies using HPLC-isolated diastereomers, as the solvents have to be evaporated and the residue should be redissolved in water. For this reason, we have now studied the separation of the respective diastereomers of ^{99m}Tc-TRODAT-1 and ^{99m}Tc-TRODAT-M and of two other ^{99m}Tc-labelled tropane derivatives on a C-18 reversed phase column using as the mobile phase mixtures of phosphate buffer and ethanol, which are more biocompatible solvents. Moreover, it was investigated whether radio-LC-MS is suitable for identification of these ^{99m}Tc-labelled agents, which are commonly obtained in pico- to nanomole quantities.

2. Materials and methods

2.1. Synthesis

Four tropane derivatives consisting of a tropane moiety coupled with a *S*-4-methoxybenzyl protected bisamino-bisthiol tetraligand have been synthesised following procedures analogous to the reported synthesis of TRODAT-1 [6,7]. Details of the syntheses will be published elsewhere. The identity of the tetraligands was confirmed by ^{1}H and ^{13}C NMR analysis and mass spectrometry. The ligands differ by the substituents on the phenyl ring attached to the tropane moiety, being, respectively, 4-Cl (TRODAT-1), 4-methyl (TRODAT-M), 2,4-dimethyl (TRODAT-DM) and 4-ethyl (TRODAT-E) (Fig. 1).

2.2. Labelling

To a labelling kit containing the lyophilized rest of 50 μg ligand (= tropane-tetraligand conjugate), 50 μg $SnCl_2.2H_2O$, 500 μg Na_2 EDTA and 20 mg NaK-tartrate in 0.5 ml phosphate buffer (pH 7, 0.5 M) was added 400–1100 megabequerel (MBq) ^{99m}Tc-pertechnetate in 0.5 ml saline obtained

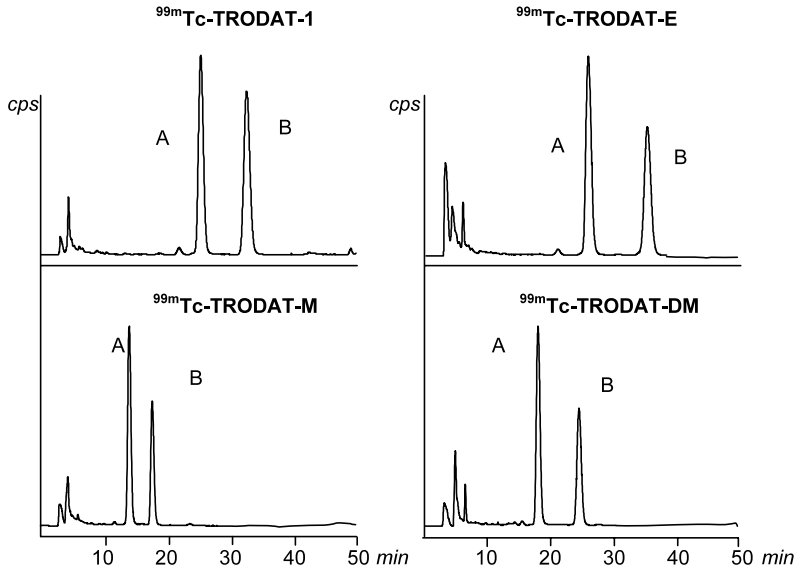

Fig. 2. HPLC-chromatograms of the labeling mixtures of the different ^{99m}Tc-labelled tropane derivatives. For details of the HPLC method, see text.

from an Ultra-Technekow ^{99}Mo/^{99m}Tc generator (Tyco Healthcare, Petten, The Netherlands), containing 150–300 pmol total technetium (^{99m}Tc+^{99}Tc). The vial was heated for 10 min at 100 °C and allowed to cool to room temperature.

2.3. High performance liquid chromatography (HPLC)

The HPLC equipment consisted of a Merck-Hitachi ternary gradient pump (model L-6200 intelligent pump, Merck, Overijse, Belgium) a Valco N6 injector (Alltech, Laarne, Belgium) and an XTerra™ RP18 (5 μm) cartridge (4.6 mm × 250 mm) (Waters, Milford, MA, USA) eluted isocratically with a mixture of ethanol–0.02 M KH_2PO_4 pH 2.5 (25:75, v/v) at a flow rate of 1 ml/min. The column effluent was monitored for radioactivity using a 2-inch NaI(Tl) scintillation detector coupled to a single channel analyzer and a Rachel analysis program (version 1.40, Lablogic, Sheffield, UK).

2.4. Resolution and symmetry

Resolution (Rs) between peaks was determined with the formula:

$$\mathrm{Rs} = \frac{1.18(t_{\mathrm{R}b} - t_{\mathrm{R}a})}{b_{0.5a} + b_{0.5b}} \quad t_{\mathrm{R}b} > t_{\mathrm{R}a}$$

$t_{\mathrm{R}a}$ and $t_{\mathrm{R}b}$ being the respective retention times and $b_{0.5a}$ and $b_{0.5b}$, the width of the peaks at half the maximum height.

The symmetry factor was determined using the formula:

$$\frac{w_{0.05}}{2d}$$

$w_{0.05}$ being the width of the peak at 1/20 of the height and d, the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20 of the peak height.

2.5. Radio-LC-MS analysis

Radio-LC-MS was carried out using a Waters Alliance 2690 separations module (Waters) an Xterra MS RP18 3.5 μ column (21 mm × 50 mm) (Waters) eluted with gradient mixtures of 0.1% ammonium formate and acetonitrile (from 20 to 80% acetonitrile (v/v) in 20 min) at a flow rate of 300 μl/min, a radiometric detector (3-inch NaI(Tl) detector connected to a radiation analyzer module, The Nucleus, Oak Ridge) and a time-of-flight

Table 1
Retention time (Rt), resolution (Rs) and ratio of both diastereomers (A and B in order of elution) of the ^{99m}Tc-labelled derivatives ($n = 3$) analyzed with the newly developed HPLC system

	R_t A	R_t B	R_s	A/B
^{99m}Tc-TRODAT-1	25′22″	32′38″	3.8	1.0
^{99m}Tc-TRODAT-M	14′09″	17′38″	3.3	1.5
^{99m}Tc-TRODAT-DM	17′45″	21′12″	5.1	1.6
^{99m}Tc-TRODAT-E	25′37″	34′04″	4.5	1.3

mass spectrometer (Micromass LCT, Manchester, UK) with electrospray ionization (ESI) in positive mode (ES+). A volume of 10 μl of the above described labelling reaction mixture was injected, corresponding to 3–6 pmol of the ^{99m}Tc-tropane derivatives.

Table 2
Symmetry factor of both diastereomers (A and B in order of elution) of the ^{99m}Tc-labelled derivatives ($n = 3$) in the newly developed HPLC system

	Peak A	Peak B
^{99m}Tc-TRODAT-1	1.13	1.10
^{99m}Tc-TRODAT-M	1.11	1.17
^{99m}Tc-TRODAT-DM	1.16	1.12
^{99m}Tc-TRODAT-E	1.15	1.09

3. Results and discussion

Different solvent mixtures were examined as mobile phase for the separation of the diastereomers of ^{99m}Tc-labelled tropane derivatives on an XTerra column. HPLC using gradient mixtures of ammonium acetate buffer (0.1 M) and acetonitrile,

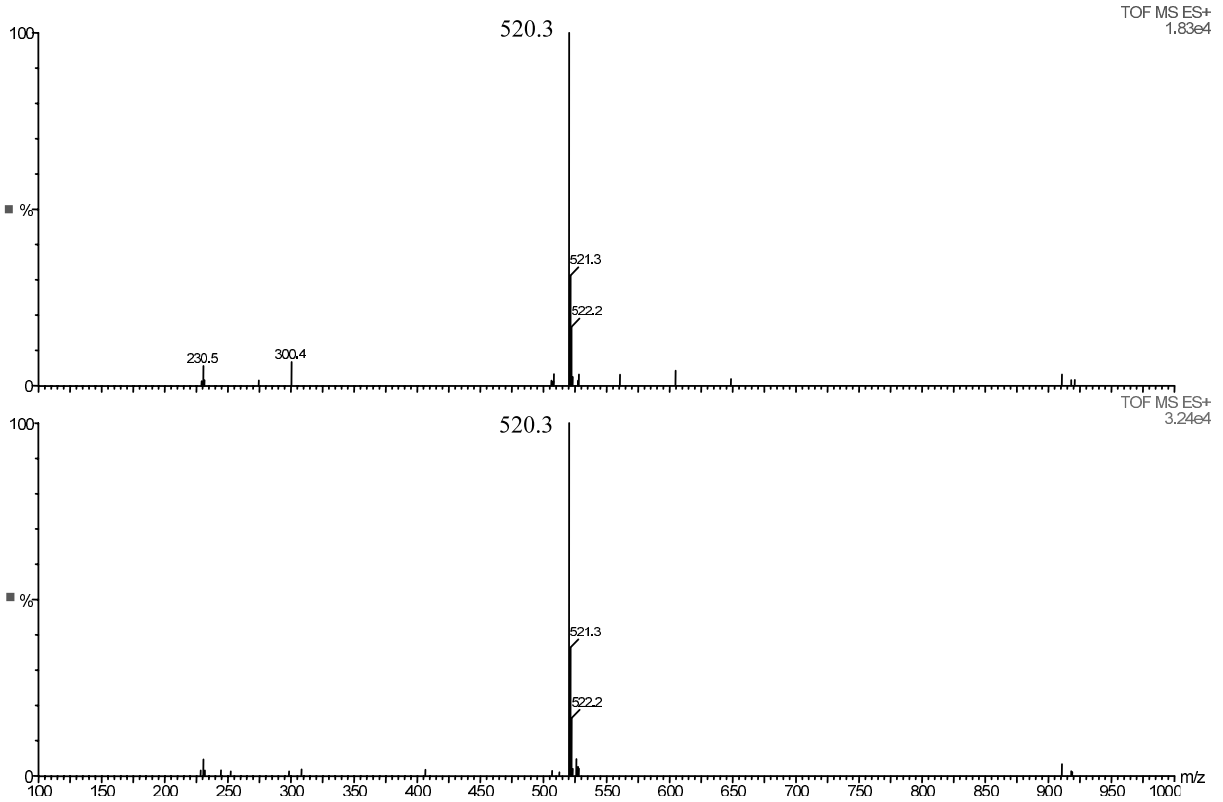

Fig. 3. Summed mass spectra (corrected for background) over each of the two main peaks observed on the radiometric channel in the reaction mixture after labeling of TRODAT-M (top spectrum corresponds to peak retention time (Rt) 10.47 min, bottom spectrum corresponds to peak Rt 11.21 min).

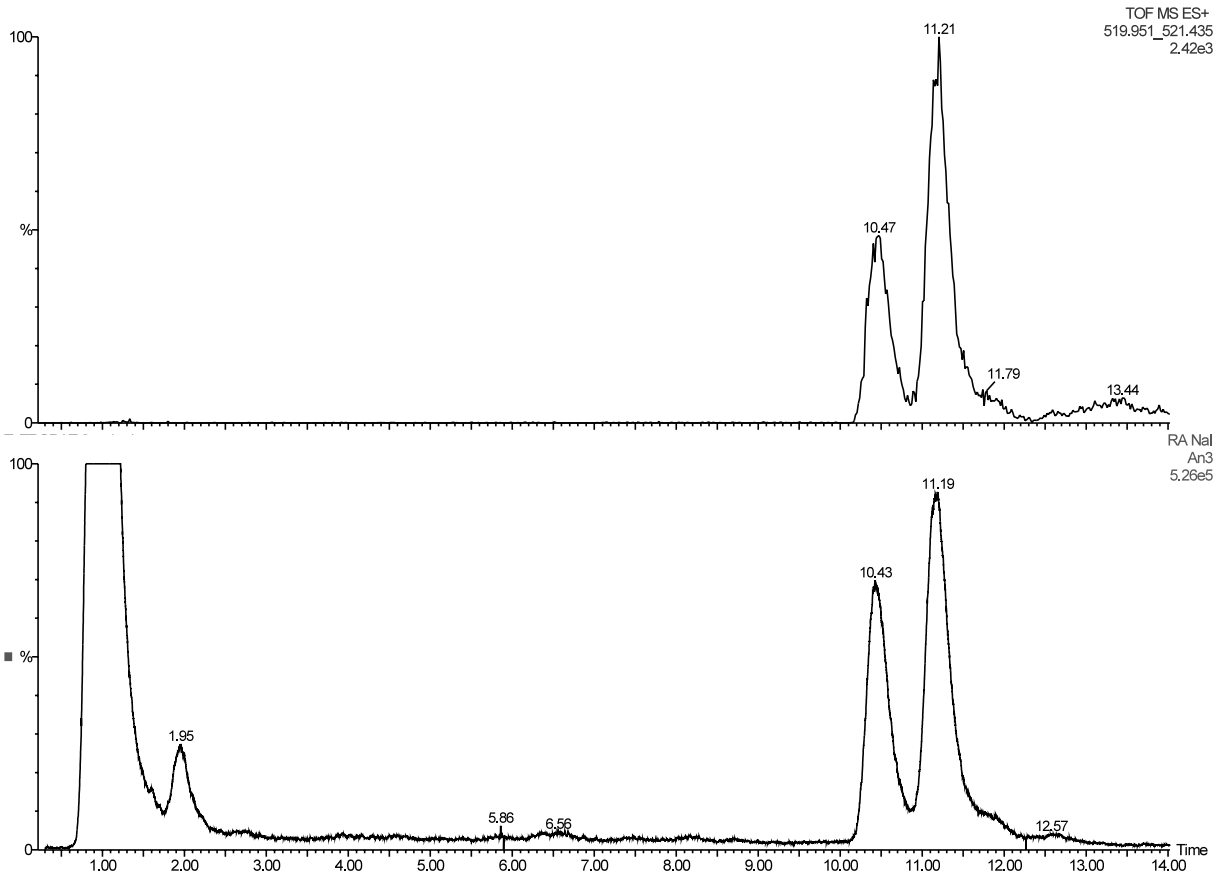

Fig. 4. Single mass chromatogram (519.951–521.435 kDa) (top) corresponding to the mass of ^{99m}Tc-TRODAT-M and the radiometric chromatogram (bottom) of ^{99m}Tc-TRODAT-M.

mixtures of trifluoroacetic acid in water (0.1% m/v) and trifluoroacetate in acetonitrile (0.1% m/v) or mixtures of neutral phosphate buffers (0.02 M) and ethanol did not result in a satisfying separation of the diastereomers of the ^{99m}Tc-labelled tropane derivatives. However, using an isocratic system with KH_2PO_4 buffer (pH 2.5, 0.02 M)–ethanol, (75:25, v/v) as the mobile phase, the chromatograms of the labelling reaction mixtures of the different ^{99m}Tc-labelled tropane derivatives all showed two well separated peaks (Fig. 2) apart from some polar radiolabelled impurities. The resolution of the separated assumed diastereomers of the different complexes on this HPLC system was more than three (Table 1) and the peaks showed only slight tailing as the symmetry factor was close to one (Table 2). As the solvents used for HPLC are compatible with biological experiments, the isolated derivatives were directly suitable for intravenous injection after dilution and aseptic filtration and evaporation of organic solvents can be avoided if the newly developed HPLC method is used.

Radio-LC-MS analysis of the labelling reaction mixtures showed for each of the investigated compounds a background subtracted mass spectrum with a peak corresponding to the theoretical molecular ion mass of the proposed structures of the complexes. As an example, the mass spectra of both diastereomers of Tc-TRODAT-M are presented in Fig. 3.

Accordingly, the single ion mass chromatograms of the mass interval corresponding to the theoretical molecular ion mass largely parallels the

radiometric chromatogram. As an example, the single ion mass chromatogram and the radiometric chromatogram of the radiolabelling mixture of ^{99m}Tc-TRODAT-M are displayed in Fig. 4. Both chromatograms show two peaks with an identical retention time and a similar shape.

Radio-LC-MS analysis after injection of 3–6 pmol of a Tc-TRODAT derivative, thus allowed to confirm that the two main ^{99m}Tc-labelled components in the examined radiolabelling mixtures have a molecular mass corresponding with the presumed structure. The results also strongly support the assumption that in each of the four mixtures, the two main peaks correspond to diastereomers.

4. Conclusion

A convenient and practical HPLC-method on an RP18 column for purification and isolation of the diastereomers of ^{99m}Tc-TRODAT-1 and three of its derivatives has been developed. The mobile phase in the newly developed method (mixture of aqueous buffer and ethanol) is compatible with biological studies. The high sensitivity of radio-LC-MS allowed the confirmation of the molecular ion mass and thus indirectly the identity of the studied ^{99m}Tc-labelled tropane derivatives. The data obtained support the assumption that the two main radiolabelled species in each of the reaction mixtures are diastereomers.

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